Supporting Information

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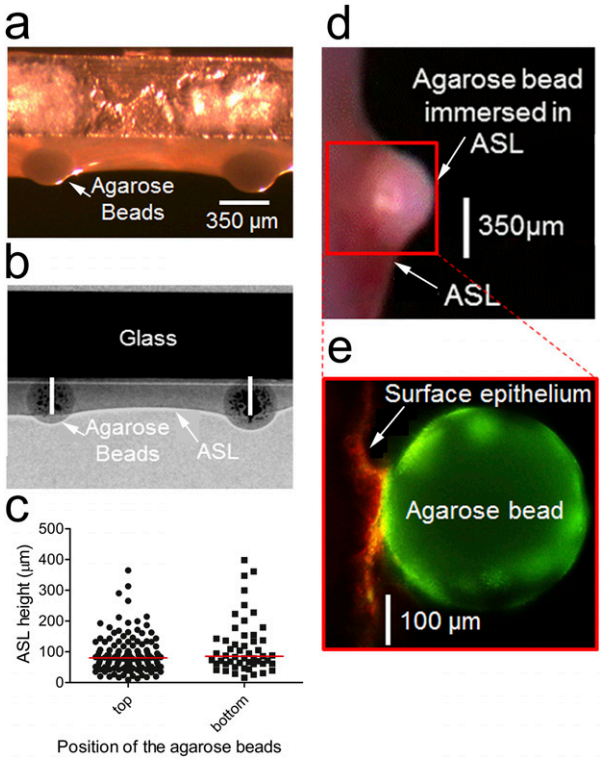

Fig. S1. Agarose beads come into direct contact with the surface epithelium. The agarose beads that we use for measuring airway surface liquid (ASL) layer height must be positioned at either the top or the bottom of the preparation, where the ASL–air interface is parallel to the X-rays reaching the sample. The position of the beads in the ASL depends on the forces acting on them (i.e., gravity, surface tension, and buoyancy); however, buoyancy is a minor force acting on the beads, because the beads are loaded with X-ray–absorbing salts with high molecular weight, and the density of the beads exceeds that of the ASL (1.3 g cm^{-3} vs.1 g cm^{-3}) (44). As a result, the position of the beads is determined largely by gravity and surface tension. Beads located at the bottom of the preparation are subject to both gravity and surface tension pushing them against the tissue. For beads located at the top of the preparation, gravity and surface tension will have opposing effects, with surface tension pushing the beads against the tissue and gravity pulling away from the tissue. (*A* and *B*) Mathematically calculating whether surface tension will be sufficient to push the beads against the tissue is difficult; thus, we directly tested whether the surface tension of the ASL is sufficient to force the beads located at the top of the preparation up against the tissue. To this end, we placed ASL collected from swine trachea on the underside of a cover glass and immersed agarose beads in it, then imaged the preparation with light microscopy (*A*) and synchrotron-based phase-contrast imaging (*B*). In this preparation, gravity is pushing the beads away from the cover glass, and surface tension of the ASL is pulling them toward the glass. The images show that the beads are touching the glass cover, indicating that surface tension is greater than gravity. To further test whether the force of gravity affects the position of the beads in the ASL, and thus the ASL height measured, we compared the ASL height measurements obtained from beads placed at the top of the preparation and those placed at the bottom. If the force of gravity was pushing the beads away from the epithelium, we would expect to see smaller ASL height measurements obtained from beads located at the top of the preparation compared with those obtained from beads at the bottom of the preparation. Our analysis found no significant differences in ASL measurements from beads at the top and beads at the bottom. (*C*) Scatterplot and median ASL height measured with beads placed at the top and bottom of the preparation ($n = 132$ for top and $n = 51$ for bottom; $P = 0.19$, Mann–Whitney test). Thus, the results suggest that the force of gravity does not influence our measurements (i.e., ASL surface tension is greater than gravity) and that the measured height of the ASL is not dependent on the position of the beads. We performed a final test of whether the beads are in direct contact with the surface epithelium by labeling the beads with FITC and labeling the surface epithelium with Rhod-2-AM. A piece of trachea was dissected, as described for secretion assays. The mucosal side was incubated in PBS containing Rhod-2-AM (50 μM) and pluronic F-127 (0.1%) for 30 min, then washed with PBS and dried with a stream of air while the serosal side was incubated in Krebs solution. The preparation was then stimulated with carbachol (1 μM on the serosal side) for 20 min, to stimulate ASL secretion by the submucosal glands. The FITC-labeled agarose beads were then placed in the ASL, and the preparation was viewed under a two-photon excitation microscope. (*D*) With the aid of a dissecting microscope, we verified that the beads were immersed in the ASL. (*E*) The two-photon microscope images showed that the beads (green) and surface epithelium (red) were in direct contact (yellow, $n = 47$ repetitions).